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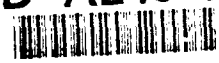

US AIR FORCE FUNDED STUDY OF

HYDRAZINE METABOLISM AND TOXICITY

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Dr J.A. TIMBRELL,
Toxicology Unit, School of Pharmacy,
University of London.

Final Report. December 1991.

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The research project has revealed the following:

1. The uptake of hydrazine into the liver may be a saturable process.
2. After a hepatotoxic dose of hydrazine the concentration in the liver is about 0.2 mM.
3. Some hydrazine remains in the liver 24 hours after a single dose at a level higher than the plasma level.
4. At the highest dose level (81 mg/kg) rats lost weight over the following 4 days and liver weight was decreased. At a dose of 27 mg/kg, the liver weight was elevated 4 days after the single dose.
5. The rats given the highest dose still showed fatty liver 4 days after dosing. Rats given lower doses also showed some vacuolation in hepatocytes.
6. Determination of hydrazine and acetylhydrazine in urine after various single doses showed a dose dependent decrease in acetylation.

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7. NMR revealed a number of metabolites: unchanged hydrazine, acetyl and diacetylhydrazine, hydrazones with pyruvate and 2-oxoglutarate, urea and ammonia.
8. Studies in hepatocytes *in vitro* have shown that hydrazine is cytotoxic at concentrations of 16 mM and above.
9. Hepatocyte studies *in vitro* have also shown that 8 mM hydrazine depletes ATP and glutathione.
10. Hydrazine is metabolised by microsomal enzymes *in vitro*. Cytochrome P-450 is responsible for some of this metabolism but other enzymes may also be involved.
11. This metabolism can be inhibited by cytochrome P-450 inhibitors and pretreatment of animals with phenobarbital increases metabolism *in vitro*.
12. Hydrazine is also metabolised by mitochrondrial enzymes *in vitro*.
13. Hydrazine inhibits the formation of ATP in isolated mitochrondria *in vitro*.
14. The metabolism of hydrazine by microsomal enzymes in vitro is increased by pretreatment of rats with phenobarbital but not by pretreatment with β-naphthaflavone, acetone or isoniazid.
15. Human liver microsomes metabolise hydrazine at a similar rate to rat liver microsomes.
16. The presence of glutathione had no effect no the metabolism of hydrazine by rat liver microsomes.
17. Hydrazine caused a dose dependent increase in liver triglycerides *in vivo* detectable at 6 hrs and after a dose of 3 mg/kg at 9 hrs.
18. Hydrazine caused a dose dependent decrease in hepatic ATP *in vivo* detectable at 6 hrs and after a dose of 3 mg/kg at 9 hrs.
19. Hydrazine caused a dose dependent decrease in hepatic glutathione *in vivo* detectable at 6 hrs after a dose of 5 mg/kg.
20. Pretreatment of rats with microsomal enzyme inducers or inhibitors alters the hepatoxicity of hydrazine as measured by accumulation of triglycerides : phenobarbital and β-naphthaflavone pretreatment decrease toxicity; acetone and isoniazid increase toxicity; piperonyl butoxide increases hepatotoxicity. Depletion of hepatic ATP and glutathione were not significantly affected.
21. Single doses of hydrazine caused a dose related decrease in nitrophenol hydroxylase and ethoxyresorufin O-deethylase but had no effect on pentoxyresorufin O-deethylase, cytochrome P450, cytochrome b_5 or cytochrome P450 reductase.
22. Hydrazine administered to rats at a level of 2.5 mg/kg in the drinking water caused significant biochemical effects. Triglycerides and glutathione were decreased after exposure for 10 days. Cytochrome P450 and b_5 were increased after 10 days. The most significant change was an increase in the activity of p-nitrophenol hydroxylase, detectable after 1 day and increased several fold after 10 days.
23. Exposure to 0.25 mg/kg hydrazine in the drinking water did not cause any significant effects on the parameters measured.
24. Studies with isolated rat hepatocytes in suspension have shown that hydrazine causes a number of biochemical changes. As well as ATP depletion and glutathione depletion there is a rapid decrease in succinate dehydrogenase activity. NADPH, NADH and NAD levels in hepatocytes are all decreased at the highest concentrations of hydrazine (16 and 20 mM). Alanine and lactate levels in the hepatocytes are also elevated by hydrazine and β-hydroxybutyrate levels are decreased.
25. Isolated hepatocytes in culture also showed a depletion in ATP and cytotoxicity when exposed to hydrazine at similar concentrations to those effective in cells in suspension. Measurement of protein synthesis revealed that this was a particularly sensitive parameter being significantly inhibited by 0.5 mM hydrazine.

EOARD-TR-92-03

This report has been reviewed and is releasable to the National Technical Information Service (NTIS). At NTIS is will be releasable to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

JEFFREY C. WIGLE, Capt, USAF, BSC
Chief, Life Sciences

RONALD J. LISOWSKI, Lt Col, USAF
Chief Scientist

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OVERALL SUMMARY

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3. Some hydrazine remains in the liver 24 hours after a single dose at a level higher than the plasma level.

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6. Determination of hydrazine and acetylhydrazine in urine after various single doses showed a dose dependent decrease in acetylation.

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25. Isolated hepatocytes in culture also showed a depletion in ATP and cytotoxicity when exposed to hydrazine at similar concentrations to those effective in cells in suspension. Measurement of protein synthesis revealed that this was a particularly sensitive parameter being significantly inhibited by 0.5 mM hydrazine.

INTRODUCTION

Hydrazine is an important chemical substance being used extensively in industry, as a rocket fuel and it is a metabolite of two widely used drugs (Timbrell and Harland, 1979; Blair *et al.* 1984).

We have previously studied hydrazine toxicity and metabolism (Wright and Timbrell, 1978; Timbrell *et al.* 1982), recently using the technique of high resolution NMR (Sanins *et al.* 1988; 1990; Preece *et al.* 1991a,b). We have shown that hydrazine does not cause lipid peroxidation (Preece and Timbrell, 1989) and causes depletion of ATP in the liver after hepatotoxic doses (Preece *et al.* 1990).

The object of this project was to study the metabolism and disposition of hydrazine *in vivo* and *in vitro* in relation to the hepatotoxicity.

The funding for the project supports a graduate research student who is working full time on the project. A postdoctoral worker and a graduate research student funded from other sources have also contributed to some of the work in this report.

The experimental details for the studies are given in 1st and 2nd annual reports.

METHODS

The effect of hydrazine on various biochemical parameters was determined after a single i.p. dose and after repeated exposure. Repeated exposure to hydrazine was carried out by putting hydrazine in the drinking water and monitoring intake. Two exposure levels were used, 2.5 and 0.25 mg/kg/day.

RESULTS

Results can be divided into :

1) Metabolic studies by NMR
2) In vivo disposition
3) In vitro studies
4) Hepatocyte studies
5) In vivo biochemical effects

Since the 2nd annual report the following results have been obtained.

In vivo Studies
Single doses of hydrazine as low as 3 mg/kg cause a significant elevation of liver triglycerides 9 hours after dosing and a significantly decreased liver level of ATP. Although affected, liver glutathione was not signficantly depleted by this dose. (For all three parameters changes are dose related). β-Naphthaflavone pretreatment of rats prior to treatment with a single dose of hydrazine caused a significant reduction in the accumulation of triglycerides. There was no significant effect on glutathione or ATP levels.

Pretreatment of rats with isoniazid prior to a single dose of hydrazine resulted in a significant increase in triglyceride accumulation. Glutathione and ATP depletion were not significantly increased.

There was no dose related effect of single i.p. doses of hydrazine up to 60 mg/kg on either cytochrome P450 or cytochrome b_5 content of liver microsomes. There was some effect on NADPH cytochrome P450 reductase level but it was not dose related. However a single dose of hydrazine did affect some cytochrome P450 activities. Thus nitrophenol hydroxylase (NPH) and ethoxyresorufin O-deethylase (EROD) activity showed a dose related decrease. However pentoxyresorufin O-deethylase (PROD) activity was not significantly affected.

The effect of continuous hydrazine exposure via the drinking water (2.5 mg/kg) on a variety of biochemical parameters was studied. Liver triglyceride level was initially raised but was decreased at 10 days and hepatic glutathione was decreased at all time points up to 10 days.

Continuous exposure to hydrazine for 10 days results in fluctuations in and finally an increase in cytochrome P450 and cytochrome b5 content. There were also fluctuations in EROD and PROD and in NADPH cytochrome P450 reductase activity.

The most significant and interesting observation however was the dramatic increase in nitrophenol hydroxylase activity. This activity was significantly increased after 1 day and by 10 days was increased by at least 3 times.

The lower exposure level of hydrazine (0.25 mg/kg/day) resulted in small changes in the biochemical parameters measured but these are not felt to be biologically significant.

DISCUSSION

Overall the project has explored the disposition, metabolism and biochemical effects of hydrazine. The objective was to understand the hepatic toxicity in terms of biochemical changes and relate it to the metabolism of hydrazine. The use of proton and ^{15}N NMR has given us significant insight into the metabolism of hydrazine. It revealed that unchanged hydrazine is excreted in significant amounts, that acetylhydrazine and diacetylhydrazine are excreted and that hydrazones are definitely formed. Previous analytical techniques had only indirectly measured these metabolites. The toxicological importance of the formation of a cyclised stable hydrazone with 2-ketoglutaric acid is not yet known. However, as this is a significant metabolite, it may be that its formation depletes the level of 2-ketoglutarate *in vivo*. Using gas chromatography/mass spectrometry the disposition of hydrazine after toxic and non toxic doses was examined. From this data the level of hydrazine in the liver and plasma after these doses was determined. The data indicated that hydrazine uptake by the liver is not simple diffusion but may be a saturable uptake system. This could have implications for repeated low level exposure especially as our data showed that hydrazine is still detectable in the liver 24 hours after a single dose.

Study of the biochemical effects of acute doses of hydrazine revealed several perturbations could be detected both in rats *in vivo* and in isolated hepatocytes *in vitro*. Thus ATP and glutathione were depleted in a dose dependent manner and as previously described there was a dose dependent accumulation in triglycerides *in vivo*. Accumulation of triglycerides was also detected in preliminary experiments *in vitro*. In isolated hepatocytes in culture hydrazine caused a dose dependent inhibition of protein synthesis, ATP depletion and cytotoxicity. The inhibition of protein synthesis was the most sensitive indicator of hydrazine exposure and may underlie the accumulation of triglycerides, although the ATP depletion may also be involved. Also in isolated hepatocytes the enzyme succinate dehydrogenase was found to be inhibited at lower concentrations of hydrazine than were cytotoxic. The inhibition of this mitochondrial enzyme may reflect the damage to this organelle that has been described by electron microscopy after exposure to hydrazine.

The effect of pretreatments which may influence hydrazine metabolism on some of these biochemical changes indicates that metabolism may be important. Thus inhibition of the microsomal enzyme system increases and induction with phenobarbital decreases triglyceride accumulation. However induction with acetone and isoniazid treatment also increases the hepatotoxicity. Thus it seems likely that hydrazine toxicity is mediated via a metabolite produced by the specific isozyme of cytochrome P450 induced by isonazid and acetone (P450IIE1). *In vitro* studies in rat liver microsomes prepared from pretreated rats did not show an increase in overall metabolism of hydrazine however. This may reflect the fact that the assay measures disappearance rather than the production of specific and as yet unknown metabolites. Thus a change in the proportion of different metabolites produced would not be detected. Further work is necessary both *in vivo* and *in vitro* to determine which metabolic pathway is involved in the toxicity. Therefore the *in vivo* finding that repeated low level exposure to hydrazine in the drinking water induced the activity of the same isozyme of cytochrome P450 as acetone and isoniazid is of particular interest and significance. This means that chronic exposure to hydrazine may increase its own toxicity. Furthermore exposure to hydrazine in the drinking water also led to a decrease in hepatic glutathione levels which may increase susceptibility. This is an important area for future investigations both to determine the effect on toxicity and on metabolism of hydrazine. Also the time course and maximum extent of induction have not been determined. The mechanism of the induction is currently unknown but it is not due to the small decrease in food consumption observed during exposure to hydrazine.

Overall therefore hydrazine undergoes significant metabolism, part of which may be catalysed by the cytochrome P450 system. The uptake of hydrazine into the liver *in vivo* may be dose related and the compound is retained for at least 24 hours. Therefore repeated dosing may lead to greater toxicity than expected from single doses. After acute doses it has significant biochemical effects which may underlie the hepatic toxicity. Some of these effects are also seen after exposure to low doses of hydrazine administered repeatedly in the drinking water. Also under these conditions hydrazine seems to induce the cytochrome P450 activity which may be responsible for its own metabolism and toxicity. Therefore for this reason also repeated doses may be more toxic than expected from single acute doses.

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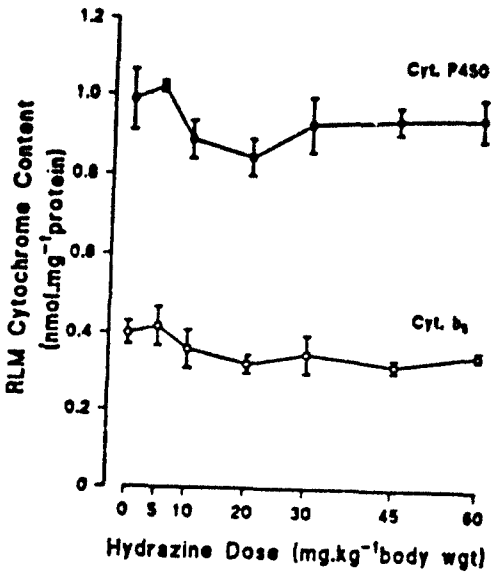
The *in vivo* HZ dose dependant effect on rat liver microsomal cytochrome P450 and cytochrome b_5

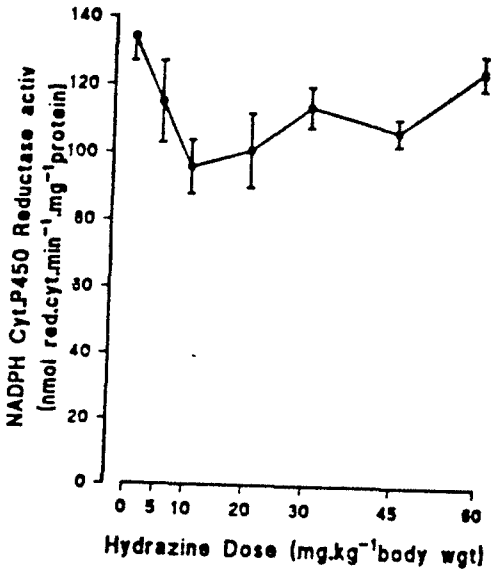


Male Sprague-Dawley rats were administered i.p. various doses of HZ and were sacrificed 6 hr later.

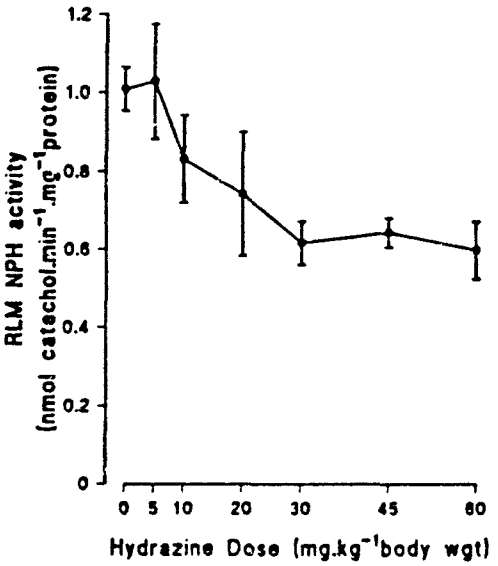
Each data point represents the mean ± SEM from three animals.
The statistical significance of each dose of HZ was assessed using:

1) Unpaired t test for individual HZ doses
   - * p<0.05
   - ** p<0.01
   - *** p<0.001

The in vivo HZ dose dependant effect on rat liver microsomal NADPH cyt.P450 reductase activity



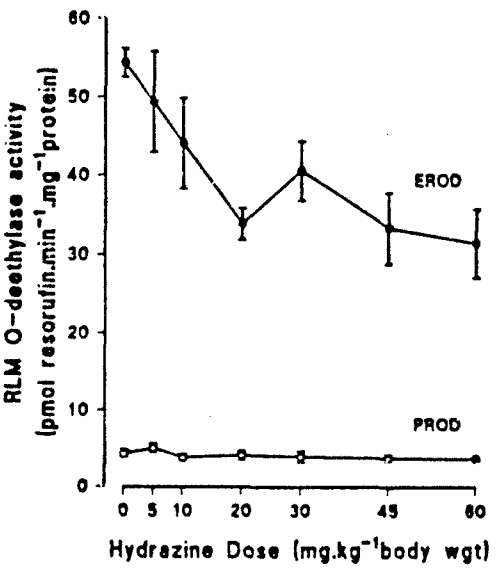
The *in vivo* HZ dose dependant effect on rat liver microsomal NPH activity



Male Sprague-Dawley rats were administered various doses of HZ and were sacrificed 6 hr later.

Each data point represents the mean ± SEM from three animals.
The statistical significance of each dose of HZ was assessed using:

The in vivo HZ dose dependant effect on rat liver microsomal EROD and PROD activity



1) Unpaired t test for individual HZ doses
   - * $p<0.05$
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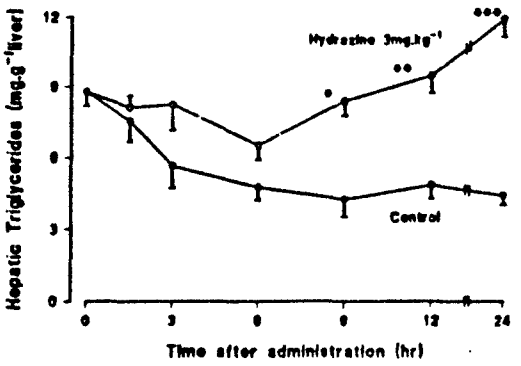
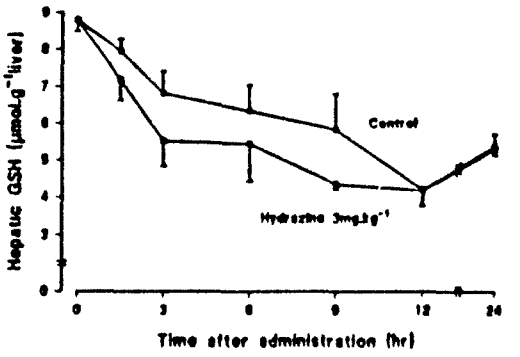
   - *** $p<0.001$

The *in vivo* time-course of rat liver triglyceride accumulation following administration of 3mg.kg^{-1}body wgt HZ



Male Sprague-Dawley rats were administered 3mg.kg^{-1} HZ i.p. (●) while control animals (O) were administered saline only.

The *in vivo* time-course of rat liver GSH depletion following administration of 3mg.kg^{-1}body wgt HZ

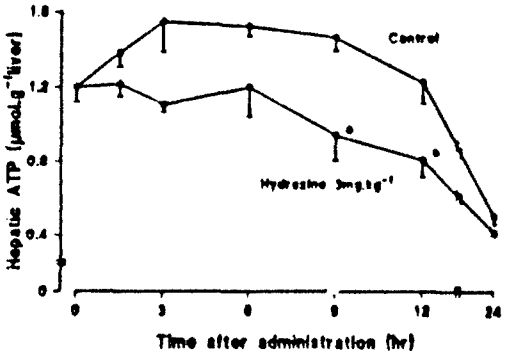


After dosing animals were starved and then sacrificed at different time intervals.

Each data point represents the mean ± SEM from three animals.

The statistical significance of HZ dosing was assessed using the unpaired t test.

The *in vivo* time-course of rat liver ATP depletion following administration of 3mg.kg^{-1}body wgt HZ



* p<0.05
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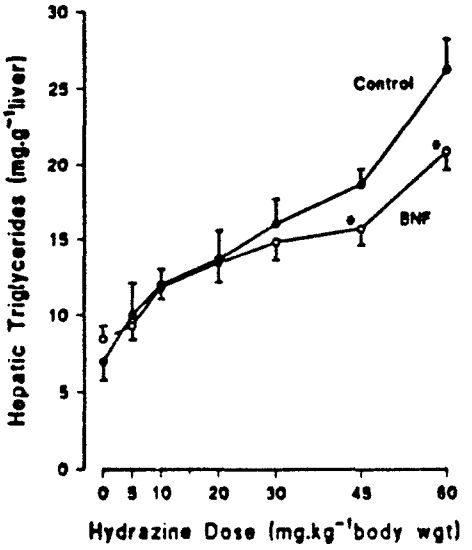
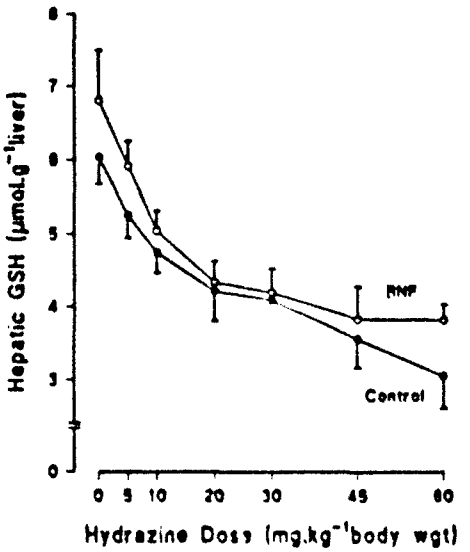
The effect of BNF pretreatment on the *in vivo* HZ dose dependent accumulation of rat liver triglycerides
(ANOVA $p<0.01$)



Male Sprague-Dawley rats were administered various doses of HZ i.p. after pretreatment with either BNF (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.
Each data point represents the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of HZ was assessed using:

The effect of BNF pretreatment on the *in vivo* HZ dose dependent depletion of rat liver GSH
(ANOVA p not significant)



1) 2 way ANOVA for shifts in HZ dose response.

2) Unpaired t test for individual HZ doses
   * $p<0.05$
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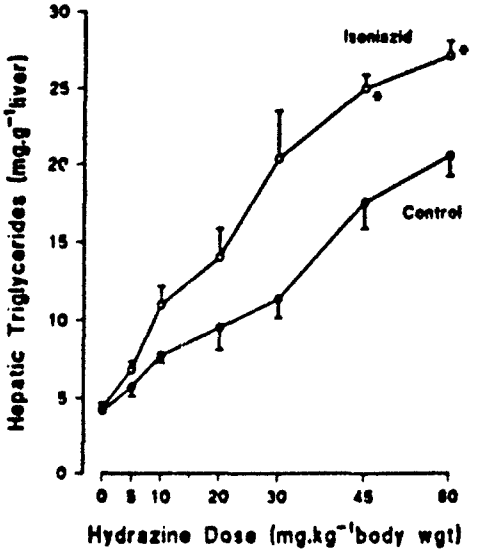
3) Comparison of AUC

The effect of isoniazid pretreatment on the *in vivo* HZ dose dependent accumulation of rat liver triglycerides
(ANOVA $p<0.001$)



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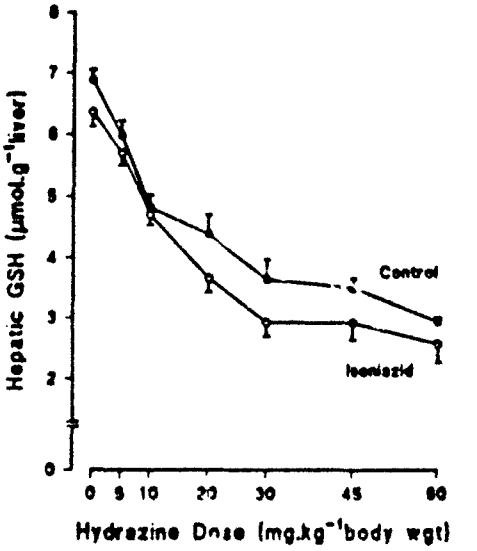

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2) Unpaired t test for individual HZ doses
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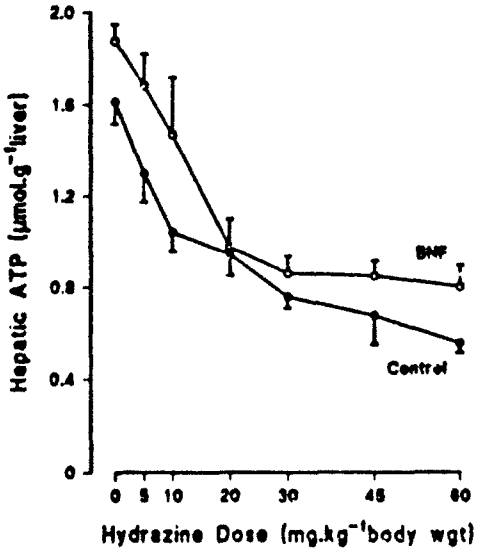
   - *** $p<0.001$

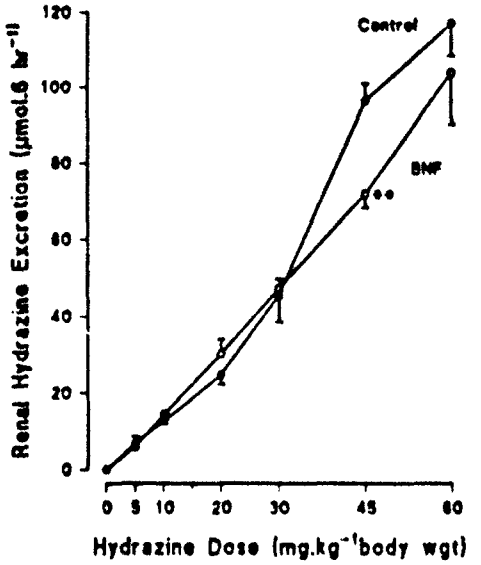
3) Comparison of AUC

The effect of BNF pretreatment on the *in vivo* HZ dose dependent depletion of rat liver ATP
(ANOVA p not significant)



Male Sprague-Dawley rats were administered various doses of HZ i.p. after pretreatment with either BNF (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.
Each data point represents the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of HZ was assessed using:

The effect of BNF pretreatment on the HZ dose dependent renal excretion of HZ in rat
(ANOVA p not significant)

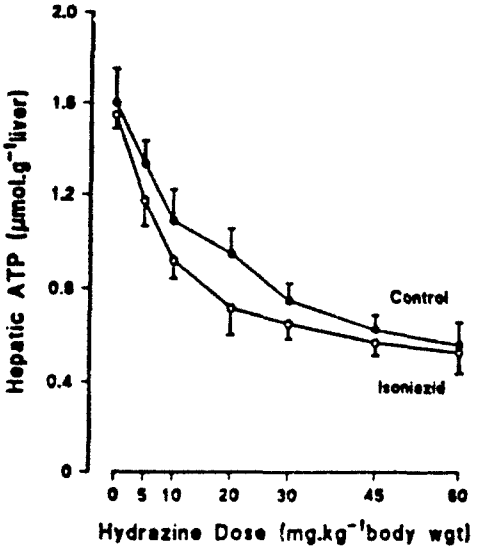


1) 2 way ANOVA for shifts in HZ dose response.

2) Unpaired t test for individual HZ doses
   - * p<0.05
   - ** p<0.01
   - *** p<0.001

3) Comparison of AUC

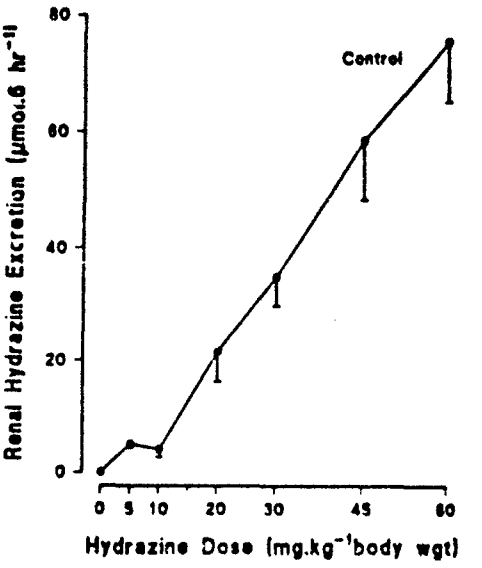
The effect of isoniazid pretreatment on the *in vivo* HZ dose dependent depletion of rat liver ATP
(ANOVA p not significant)



Male Sprague-Dawley rats were administered various doses of HZ i.p. after pretreatment with either isoniazid (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Each data point represents the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of HZ was assessed using:

The HZ dose dependent renal excretion of HZ in rat
(analysis of isoniazid pretreated urine was not possible due to assay interference)

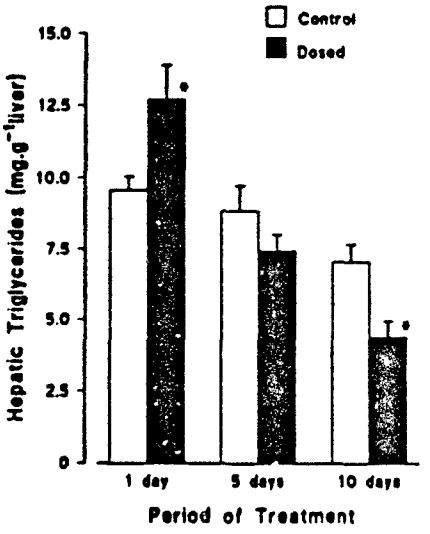


1) 2 way ANOVA for shifts in HZ dose response.

2) Unpaired t test for individual HZ doses
   - * p<0.05
   - ** p<0.01
   - *** p<0.001

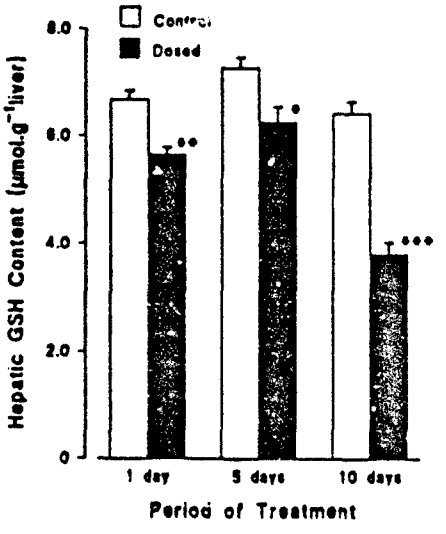
3) Comparison of AUC

The effect of 0.78mM HZ in drinking water on rat liver triglycerides



Male Sprague-Dawley rats received 0.78mM HZ in their drinking water ($2.5mg.kg^{-1}.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 1,5 and 10 days.
Each value represents the mean ± SEM from four animals.

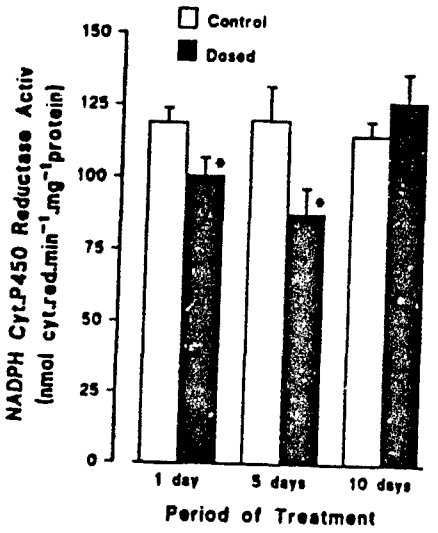
The effect of 0.78mM HZ in drinking water on rat liver GSH



The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

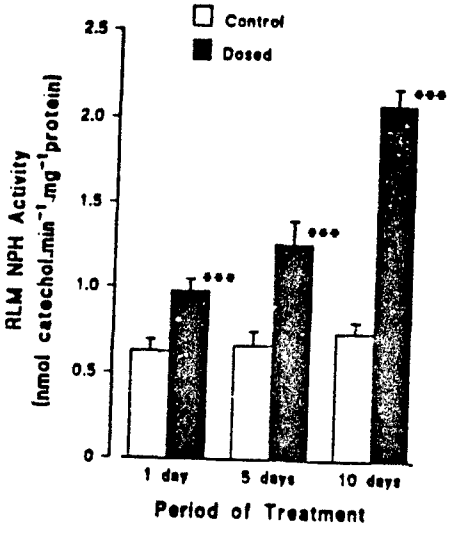
* $p<0.05$
** $p<0.01$
*** $p<0.001$

The effect of 0.78mM HZ in drinking water on rat liver microsomal NADPH cytochrome P450 reductase activity



male Sprague-Dawley rats received 0.78mM HZ in their drinking water (2.5mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 1,5 and 10 days. Each value represents the mean ± SEM from four animals.

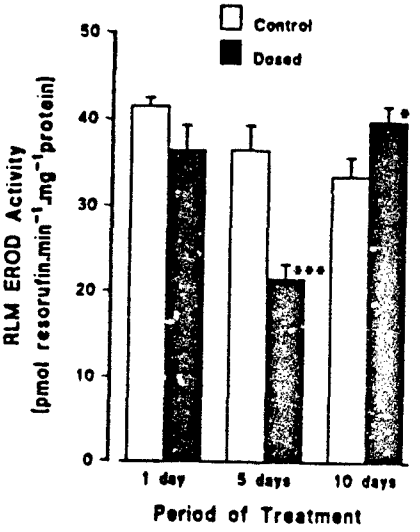
The effect of 0.78mM HZ in drinking water on rat liver microsomal NPH activity



The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

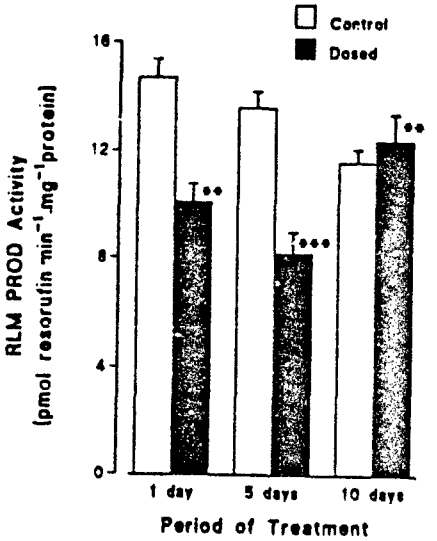
*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

The effect of 0.78mM HZ in drinking water on rat liver microsomal EROD activity



male Sprague-Dawley rats received 0.78mM HZ in their drinking water (2.5mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 1,5 and 10 days. Each value represents the mean ± SEM from four animals.

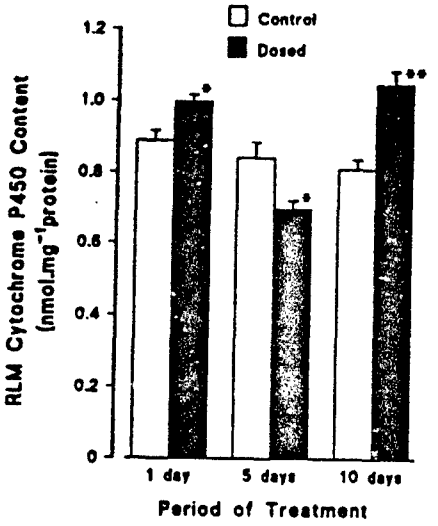
The effect of 0.78mM HZ in drinking water on rat liver microsomal PROD activity



The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

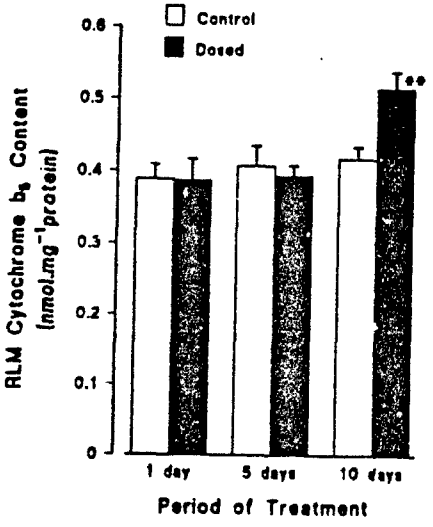
* $p<0.05$
** $p<0.01$
*** $p<0.001$

The effect of 0.78mM HZ in drinking water on rat liver microsomal cytochrome P450



Male Sprague-Dawley rats received 0.78mM HZ in their drinking water ($2.5mg.kg^{-1}.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 1,5 and 10 days.
Each value represents the mean ± SEM from four animals.

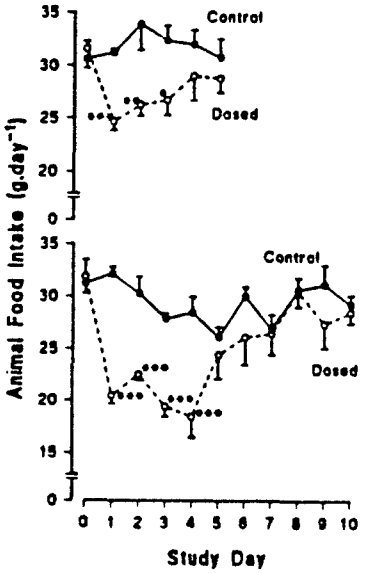
The effect of 0.78mM HZ in drinking water on rat liver microsomal cytochrome b_5



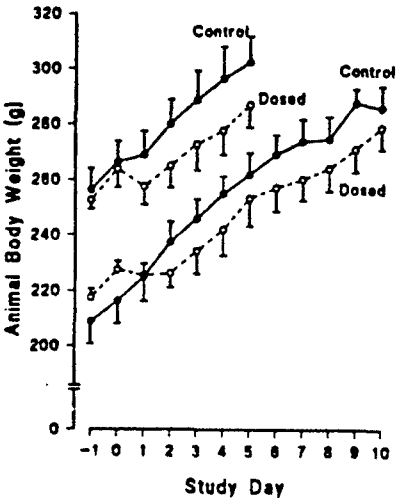
The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

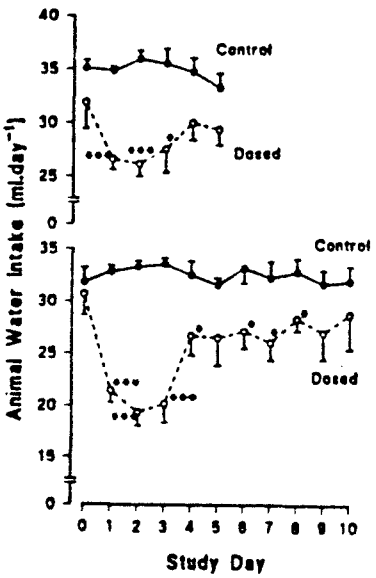
The effect of 0.78mM HZ in drinking water on daily rat food intake



The effect of 0.78mM HZ in drinking water on rat body weight



The effect of 0.78mM HZ in drinking water on daily rat drinking water intake



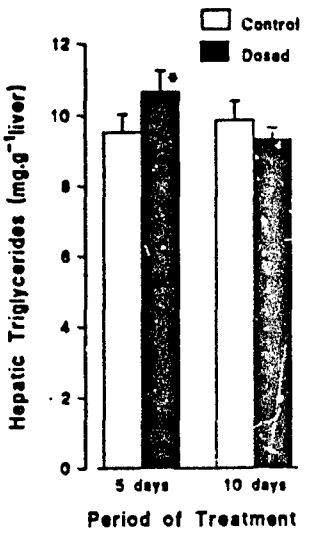
On study day 0 male Sprague-Dawley rats received 0.78mM HZ in their drinking water (O) ($2.5mg.kg^{-1}.day^{-1}$) while control animals (●) were untreated. Animals were fed *ad libitum* and were sacrificed after 5 and 10 days.

Each value represents the mean ± SEM from four animals. Drinking water and food intake was measured on each study day for the preceding 24 hr.

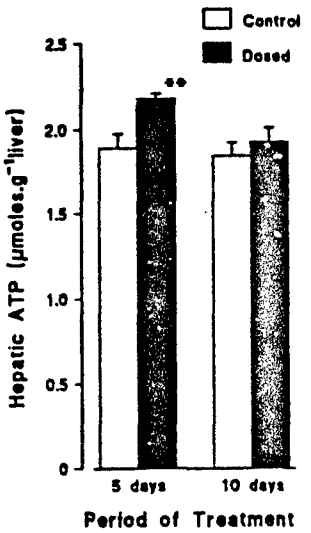
The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

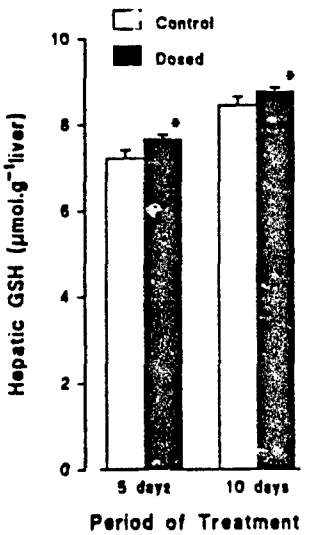
The effect of 65µM HZ in drinking water on rat liver triglycerides



The effect of 65µM HZ in drinking water on rat liver ATP



The effect of 65µM HZ in drinking water on rat liver GSH



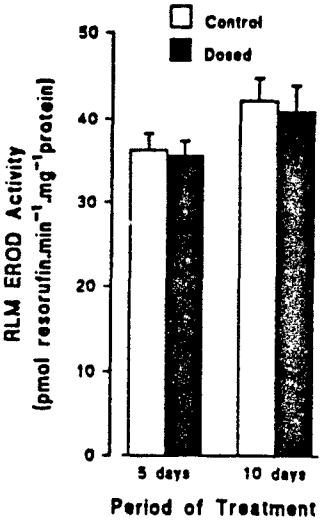
Male Sprague-Dawley rats received 65µM HZ in their drinking water ($0.25 mg.kg^{-1}.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 5 and 10 days.

Each value represents the mean ± SEM from four animals.

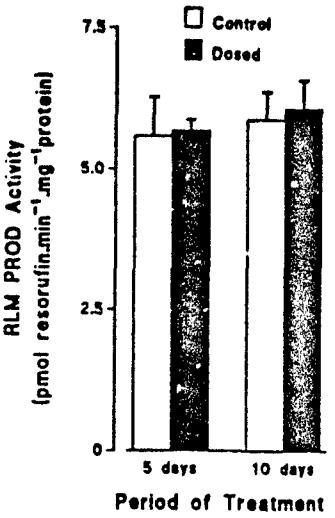
The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

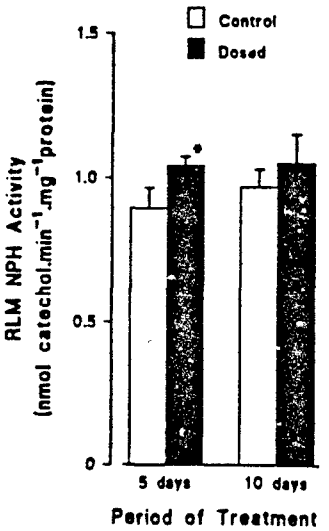
The effect of 65µM HZ in drinking water on rat liver microsomal EROD activity



The effect of 65µM HZ in drinking water on rat liver microsomal PROD activity



The effect of 65µM HZ in drinking water on rat liver microsomal NPH activity



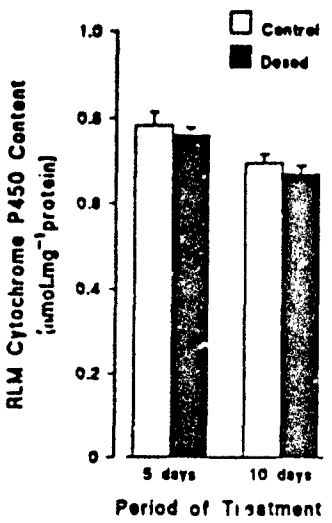
Male Sprague-Dawley rats received 65µM HZ in their drinking water ($0.25 mg.kg^{-1}.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 5 and 10 days.

Each value represents the mean ± SEM from four animals.

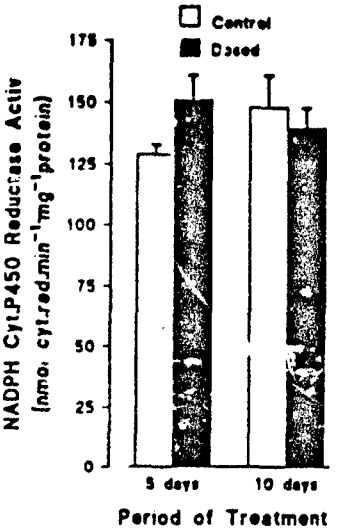
The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

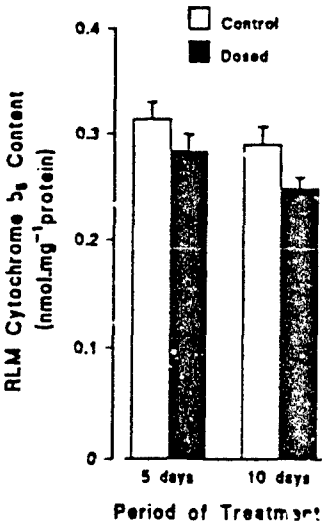
The effect of 65μM HZ in drinking water on rat liver microsomal cytochrome P450



The effect of 65μM HZ in drinking water on rat liver microsomal NADPH cyt.P450 reductase activity



The effect of 65μM HZ in drinking water on rat liver microsomal cytochrome b_5



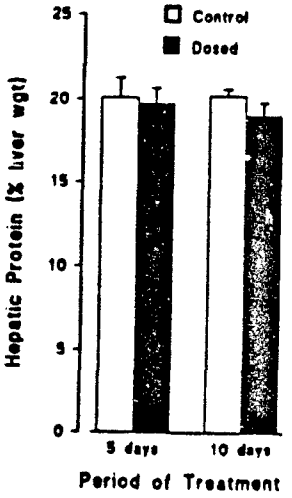
Male Sprague-Dawley rats received 65μM HZ in their drinking water (0.25mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 5 and 10 days.

Each value represents the mean ± SEM from four animals.

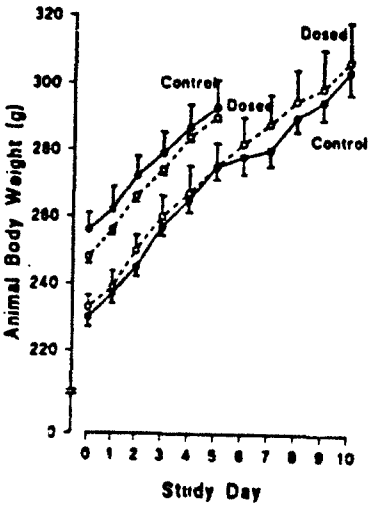
The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

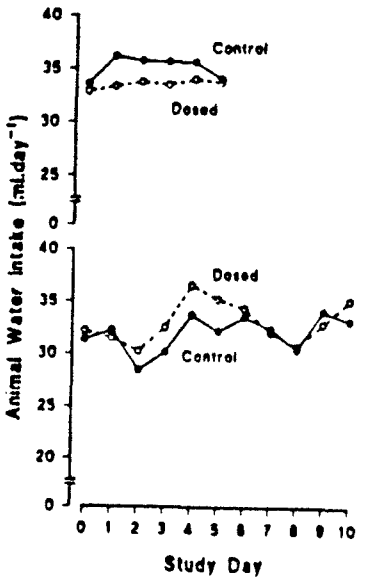
The effect of 65μM HZ in drinking water on rat liver protein



The effect of 65μM HZ in drinking water on rat body weight



The effect of 65μM HZ in drinking water on rat water intake



On study day 0 male Sprague-Dawley rats received 65μM HZ in their drinking water ($0.25 mg.kg^{-1}.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and were sacrificed after 5 and 10 days.

Each value represents the mean ± SEM from four animals. Drinking water intake was measured on each study day for the preceding 24 hr.

The statistical significance of repeated HZ dosing was assessed using the unpaired t test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$